QUANTITATIVE DETERMINATION OF MUSCLE GLYCOGEN CONTENT FOLLOWING PARTIAL HEPATECTOMY OF THE LABORATORY RAT

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INTRODUCTION AND REVIEW OF THE LITERATURE

The amazing ability of the liver to replace lost tissue rapidly and completely has resulted in a considerable quantity of research dating as early as 1833. In spite of this quantity of research being undertaken and the many directions from which it is being approached there remain several unanswered questions, the most compelling of them being: by what means is the control of regeneration effected (Bucher, 1963). The purpose of this paper is an attempt to determine whether those factors which affect the glycogen level in the liver remnant following partial hepatectomy also affects the glycogen level in the skeletal muscles during this same restorative period of time. There is the possibility that the factor causing liver tissue restoration also causes the changes in glycogen levels.

Although the term regeneration is used to refer to the process of restoration of liver tissue following damage or loss of tissue, it is an inaccurately used term and generally recognized as such by those that use it. Actual regeneration, restoration which reflects the original morphology of the liver, occurs following partial hepatectomy only in very young rats (Leduc, 1964). In mature rats a process properly described as compensatory hypertrophy and hyperplasia follows partial hepatectomy, resulting in the restoration of the liver to its original volume but with a lesser cell population and without distinctive lobes (Brues, Drury, and Brues, 1936). The lobe or lobes remaining after partial hepatectomy increase in size to compensate for the lost tissue.

The ability of the liver to "regenerate" was first postulated in 1833 by Curveilhier and in 1834 by Andral (Milne, 1909). The ensuing

studies until 1931 where "limited almost entirely to gross and microscopic examination of tissue during restoration and were at best only semiquantitative." (Brues, et al., 1936). The experimental studies in this period of time were limited almost exclusively to research with rabbits, cats and dogs.

A turning point occured in 1931 with the publication by Higgins and Anderson of a quantitative study of changes in weight of the liver of the rat following partial hepatectomy. They described a simple surgical technique for partial hepatectomy of the rat. The volume of research published thereafter would make an exhaustive bibliography of liver regeneration a project in itself.

Variation in the amount of liver tissue removed during partial hepatectomy influences the restorative process. Removal of less than the usual two-thirds results in a lesser and more gradual response; conversely more than two-thirds removal results in a predominance of cell size increase rather than an increase in the number of cells (Harkness, 1957, Glinos, 1958). Accurate weighing of the liver tissue is very difficult because the quantity of blood in the reservoir spaces of the liver cannot be precisely controlled. It is generally acknowledged, however, that the removal of the median and left lateral lobe constitutes the removal of 66 to 70% of the total liver mass in the rat (Sulkin, 1943, Harkness, 1957).

The parenchymal cell, which constitute 90 to 95% of the liver mass, display conspicuous early changes following partial hepatectomy. The earliest detectable change is the formation of vacuoles, which can be seen as early as five minutes following the operation (Steiner,

Phillips, and Miyai, 1964). By twelve hours unvacuolated cells were an extreme rarity (Davis, 1962). The vacuolation is accompanied by an immediate loss of glycogen and an accumulation of lipids, mainly neutral fats. Most observers report that the glycogen concentration is restored to one-third its normal value in 3 days and to normal by 8 days. Davis (1962), however reported a temporary re-appearance of glycogen areas at approximately 22 to 23 hours with large quantities recognized at 24 hours. Bucher (1963) also reported a temporary increase in the liver glycogen level at 24 hours. During mitosis which begins at about 24 hours the glycogen content is again depleted but not as radically as immediately following surgery.

Within the first 12 hour period there is a loss of sinusoidal villi and adhesiveness between parenchymal cells although the cells of the bile canaliculi remain unchanged. This massive disturbance of the cell membrane during the early hours following partial hepatectomy may account for much of the immediate loss of cellular glycogen (Becker and Bitensky, 1969), and appear to indicate that the responsible factor is localized. According to these authors, these changes possibly release a membrance associated enzyme adenyl cyclase that gives rise to 3'5' cyclic adenosine monophosphate and stimulates glycogenolysis.

By 12 hours the cells, nuclei and nucleoli have begun an increase in size which will continue until mitosis begins at about 24 hours. Prior to mitosis the nucleic acids show evidence of an increase. Within 6 hours after partial hepatectomy the rate of RNA synthesis begins to rise reaching a miximual rate at 24 to 30 hours. DNA synthesis occurs at 16 to 18 hours reaching its peak at approximately 25 hours,

then continuing at a lower but still elevated rate for several days. In both instances nucleotide synthesis is observed prior to RNA and DNA synthesis.

Mitotic activity follows the initiation of DNA synthesis by 6 to 8 hours. Mitosis begins at approximately 24 hours and increases rapidly at first, then slowly for the next few days.

Jaffe (1954) reported a diurnal periodicity of mitosis in the regenerating liver following partial hepatectomy with high activity in the morning and low activity at night. This diurnal periodicity is not exhibited within the first 24 hours following partial hepatectomy (Jaffe, 1954; Barbiroli and Potter, 1971). A diurnal periodicity is also cited for glycogen synthesis in normal liver with glycogen synthesis more pronounced at night and glycogenolysis increased in the morning (Ekman and Holmgren, 1949). There is a debate as to whether the diurnal periodicity is caused by an intrinsic factor or whether it simply reflects the periodicity of feeding and digestion. Regardless of the cause, the diurnal periodicity does exist and is a factor which must be considered in planning experiments.

Although the phenomenon of initial glycogen loss and eventual restoration after partial hepatectomy is reported by many researchers, little more than the modifying affect of dietary, environmental and endocrine factors is known. The determining factor in tissue restoration, its source and function remains as an eluxive factor. Several determining factors have been hypothesized within the last 15 years but only one has seemed to merit continued research. This is the hypothesis which attributes the control of tissue restoration to biochemical changes

in the blood. These biochemical changes are categorized as the humoral factor or factors.

One method to determine the presence of humoral factor is through the use of parabiotic rats, wherein one or more rats that have been partially or wholly hepatectomized share a common blood supply with a rat having an intact liver. Any changes which occur in the liver of the intact rat would be attributed to a blood-borne factor.

Results of early experiments with parabiotic rat pairs by Christensen and Jacobsen (1949) and Bucher et al. (1951) indicated a slight increase in the mitotic activity of the intact partner. When two rats of parabiotic triplets have had a partial hepatectomy removing 80 to 85% of their liver tissue, the effect in the intact rat is 10 times that found in parabiotic pairs (Bucher et al., 1951). Later research (Islami, Pack, and Hubbard, 1959; Rogers et al., 1961) failed to confirm these earlier findings resulting in a diminished publication of this type of research.

Another method of determination of humoral factor utilized by many researchers involved the injection of serum of plasma from normal or partially hepatectomized donors into normal or partially hepatectomized recipients. Although the number of researchers employing this technique is considerable the results are far to inconsistent and contradictory to allow the formulation of any clear cut conclusions. The results vary from stimulation of mitotic activity, no effect, to an inhibition of mitotic activity.

More recently Fisher et al. (1971) have resumed research with parabiotic rats. They utilized several variations with pairs of rats;

an intact rat with 1) a 70% partially hepatectomized rat, 2) an 86% partially hepatectomized rat, 3) a totally hepatectomized rat with an end-to side portacaval shunt. Instead of mitotic index this research measured DNA synthesis.

A direct correlation was found between the amount of liver removed and the extent of DNA synthesis recorded in the intact liver of the partner. The intact partner at 70% partial hepatectomy showed only a slight increase of DNA synthesis, the intact partner at 86% partial hepatectomy showed a three times increase and the intact partner at 100% hepatectomy showed a five times increase in DNA synthesis. Since the DNA synthesis was stimulated and was the greatest in the last instance the researchers concluded that there is a blood-borne factor, and that it must originate elsewhere than from the liver tissue.

The parabiotic pair with a 70% partial hepatectomy and portacaval shunt exhibited the relation of liver remnant size with its ability to utilize what Fisher et al. (1971)refers to as the portal blood factor (PBF). The liver of the intact rat responded almost as much as the intact rat paired with the 100% hepatectomized rat. On the other hand, the liver remnant in the 70% partially hepatectomized rat showed very little DNA synthesis. These results prompted the conclusion that PBF is a substance normally found within the blood and that a response is provoked when there is a disturbance between the ration of PBF and parenchymal cells. The intact partner in the latter instance is receiving the PBF from both rats by virtue of the shunted liver of the 70% partially hepatectomized partner; this accounts for the maximal response in the intact liver which has the full potential for utilization of the PBF.

The relative inactivity in the liver remnant of the partially hepatectomized rat is attributed to the diminished blood supply to this tissue.

Although they(Fisher et al., 1971) stress the need to answer the questions regarding the nature, site of origin, mechanism of action, and regulation of concentration of PBF, it would seem as if a breakthrough of considerable importance has been achieved.

The predictability of the changes in glycogen content in the liver during tissue restoration following partial hepatectomy leads to an assumption that there is some type of relationship between the two processes. That the relationship is not a direct one; glycogen change causing the restoration process is evident, but any correlation must be taken into consideration. The possibility exists that the determining factor of restoration is also the factor which causes the changes in glycogen concentrations. If such is the case it may provide direction for further research in determining the nature of the determining factor of liver tissue restoration.

This research is attempting to determine if some correlation exists between the changes of glycogen level in the liver and the glycogen level in muscle tissue following partial hepatectomy, therefore providing further supportive evidence for a blood-borne factor.

MATERIALS AND METHODS

Random bred male albino Holtzman rats weighing approximately 280 grams each were used. They were fed Purina Rat Chow and watered *ad libitum*. This diet both preceded and followed the partial hepatectomy.

Partial hepatectomy was timed to allow killing at 9:00 and 10:00 a.m. Surgery was carried out under ether anesthesia and followed

with minor variation the procedure of Sulkin (1943). An incision was made proceeding caudad from the xiphoid process of the sternum 2 to 3 cm, a length sufficient to extract the liver. The median and left lateral lobes were pressed out by applying a slight pressure to the dorsal abdominal walls, the pedicles ligated, and the lobes then excised. This constitutes a removal of approximately 68% of the liver. In closing the incision, the abdominal muscles were first sutured with thread followed by the closing of the integument with wound clips. The procedures were carried out in clean but non-sterile conditions; there was no evidence of infection. This reflects other experiences with this surgical procedure (Higgins and Anderson, 1931; Sulkin, 1943; Harkness, 1957).

The animals were killed by decapitation and a portion of the Quadriceps Femoris was removed and immediately frozen. This skeletal muscle was selected because a sample of sufficient size could be quickly excised and frozen to prevent enzymatic hydrolysis of the glycogen. One rat each was killed at 1, 2, 3, 4, 6, 8, and 10 days following partial hepatectomy. These times were selected because they relate to the significant period of activity of the liver during restoration and a period of time thereafter.

Glycogen was determined by the method of Seifter with variations as reported in Methods in Enzymology Vol. III (Colowick and Kaplan, 1957). By this method tissue was digested in hot concentrated KOH, glycogen was precipitated with ethanol, washed and reprecipitated, and finally glycogen concentration was determined with a colorimeter, using anthrone as the reagent.

The excised Quadriceps Femoris, while still frozen, was

sectioned into 12 specimens free of adipose or connective tissue. The specimens were taken from representative areas of the muscle to avoid sampling a possibly localized accumulation of glycogen as sometimes occurs in the liver (Harkness, 1952). Following is the procedure of determination;

1) Place a 25-100 mg tissue sample in a centrifuge tube containing 1 ml of 30% KOH. Determine the specimen weight and close the tube with a marble.
2) Digest the tissue by placing the tube in a boiling water bath for 20 minutes.
3) Cool, digest, add 1.25 ml of 95% ethanol.
4) Mix with stirring rod, rinse rod with 60% ethanol.
5) Bring solution to boil gently in a boiling water bath.
6) Cool and centrifuge 15 minutes at 3000 rpm.
7) Decant supernatant and drain centrifuge tube well.
8) Redissolve precipitate in 1 ml of distilled water.
9) Reprecipitate with 1.25 ml of 95% ethanol, centrifuge and drain as in step 7.
10) Dissolve precipitate in 5 ml of distilled water.
11) Immerse tubes of solution in cold water.
12) Add 10 ml of anthrone reagent from a fast flowing burette, mix by swirling
13) Cover cold tubes with glass marbles, heat for 10 minutes in a boiling water bath.
14) Cool immediately and transfer solution to colorimeter tubes.
15) Read in the colorimeter after setting at 100 with blank, read at 620nm

16) Calculate the glycogen concentration using:

$$\text{of glycogen in aliquot} = \frac{100 \times U}{1.11 \times S}$$

where U = absorbance of the unknown solution.

S = absorbance of the glucose standard.

1.11 = the factor determined for the conversion of glucose to glycogen by this equation (Morris, 1948).

A glucose standard containing 100 nanograms and a distilled water blank to be used for the calibration of the colorimeter were introduced in the procedure at step 11. The anthrone reagent was prepared by dissolving 0.2 g of anthrone in 100 ml of 95% sulfuric acid. This reagent is not stable and must be refrigerated. It should be prepared fresh every two days.

A coleman Junior Spectrophotometer was used in the colorimetric determination. The colorimeter tubes were previously matched using distilled water blanks to avoid any discrepancy resulting from unmatched tubes.

The results of the colorimetric determinations were calculated on the basis of the determination factor which takes into account the different complexity of the glycogen and glucose molecules. This result was in turn computed into milligrams of glycogen per gram of tissue.

DATA AND DISCUSSION

Glycogen contents of the Quadriceps Femoris and liver at various times after partial hepatectomy are recorded in Table 1. The values for the glycogen level in muscle tissue are the means of the 12 samples taken from an individual rat per day. The means of the glycogen level in the liver are taken from Harkness (1952) and are the results of a random

sampling of from 4 to 9 rats per day. Data from the 4th day following was not reported by Harkness, but Davis (1962) indicated that it continues a gradual increase back to normal at approximately the 8th day. Figure 1 shows the 95 percent confidence intervals around the means of the glycogen level in the muscle tissue as found in Table 1.

On day 1 the glycogen level in the muscle tissue drops to a very low point, reflecting the drop found in the liver (Table 1). On day 2, while the glycogen level in the liver is dropping as a result of increased mitotic activity in the liver tissue, the glycogen value exhibits a slight increase in the muscle tissue and remains below the level found in normal rats. On days 3 and 4, when the liver glycogen level is beginning to increase slightly, the values for muscle glycogen rise to a point that may be higher than that in normal muscle. Thereafter the glycogen content returns to the normal value.

The general pattern of the changes in glycogen content of the muscle tissue following partial hepatectomy is found to be similar to those occuring in the remaining liver tissue. Although the sequence is similar, the times concerned differ within the pattern. After a rapid decrease in glycogen content in both tissues by the 1st day, the muscle tissue is beginning to show recovery by the 2nd day and continues to recover abruptly to normal and above during the 3rd and 4th days before retruning to a normal range by the 6th day. The liver glycogen content on the other hand remains low through the 4th day before being gradually restored as early as 8 days (Davis, 1962) or as long as 15 days (Harkness, 1957). In either case the factors controlling the glycogen content in the two tissues are apparently different. It is

TABLE 1. Glycogen levels in muscle tissue and liver of individual rats at selected times after partial hepatectomy (mg/g tissue).

		TIME IN DAYS						
	Normal	1	2	3	4	6	8	10
Mean level of glycogen in muscle tissue	.129	.054	.077	.144	.181	.136	.148	.123
Mean level of glycogen in liver tissue (Harkness, 1952)	38	17	11	13	Increased to normal by 8 days			

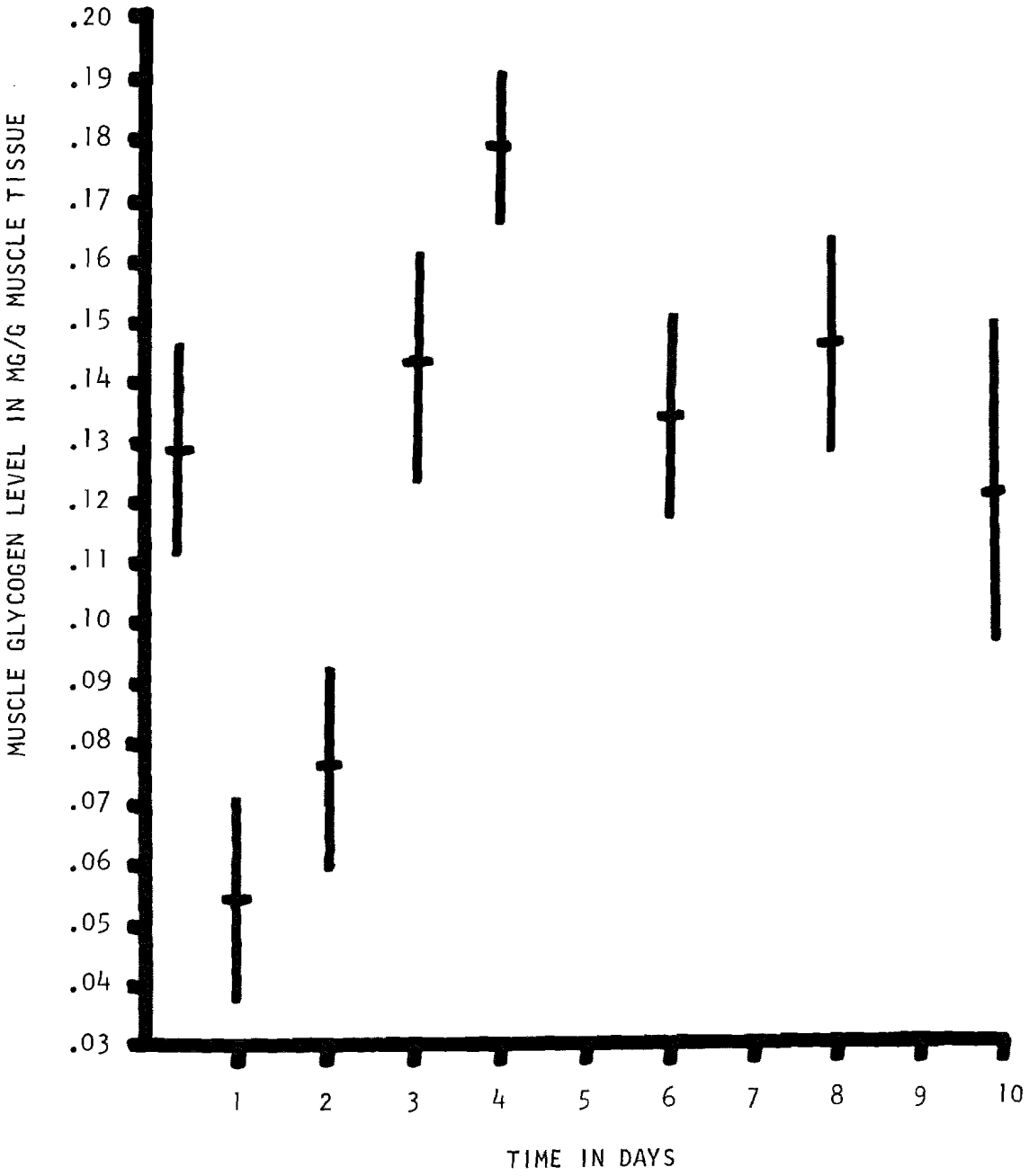

Fig. 1. Glycogen levels in muscle tissue of individual rats at selected times after partial hepatectomy. Bars indicate confidence interval of the mean of multiple samples from single rats for each time after operation.

evident that the muscle glycogen controlling factor must be blood borne inasmuch as it is a distant response to a localized tissue shock. The immediate factor known to be necessary for glycogenolysis within muscle tissue is epinephrine (Ruch and Patton, 1965) but whether the presence of the epinephrine is the result of a hypoglycemic condition or prompted by a regenerative agent remains as an unanswered question. Although much has been published regarding the blood factors responsible for the liver regeneration the emphasis has been upon the blood proteins (Glinos, 1958) with nothing directly available regarding the blood glucose level following partial hepatectomy.

Becker and Betinsky(1969) studied the affects of partial hepatectomy on the production of the hormones glucagon and epinephrine. These hormones are responsible for the activation of the enzyme adenyl cyclase whose presence causes an increase in the intracellular concentration of 3'5' cyclic adenosine monophosphate. Adenyl cyclase is membrane related and may be released from the liver parenchymal cell membranes as the cells undergo hypertrophy and hyperplasia during tissue restoration. The 3'5' cyclic adenosine monophosphate causes an enzymic activation of glycogen phosphorylase and the concomitant inactivation of glycogen synthetase. The glycogen then undergoes phosphorolysis with glucose being made available for energy utilization. Glycogenolysis within the liver can be activated by either hormone but only epinephrine can activate this response within muscle tissue (Ruch and Patton, 1965). Although the conclusion of Becker and Betinsky was that there was no significant change in the hormone levels during the first 24 hours, (the duration of their testing), the epinephrine level

was increased by 10% over control conditions. Ruch and Patton (1965) did not give specific values but did say that extremely small amounts of epinephrine, less than enough to cause a change in blood pressure, could effect a change in blood glucose content as a direct result of glycogenolysis. Since the epinephrine level was increased at the 24 hour period, and since it is the hormone primarily activating glycogenolysis within muscle tissue, the continued increased epinephrine level may be responsible for the lowering of the glycogen level in the muscle tissue. Its residual effect may also account for the slow recovery rate from day 1 to day 2 (Fig. 1). Further research would be necessary to determine 1) if a 10% increase in epinephrine during the metabolic demand of restoration could cause the observed decrease of glycogen within the muscle tissue and 2) if the increased amounts of epinephrine persist beyond the 24 hour period, and if so, how long.

If this epinephrine level could cause glycogenolysis in muscle tissue and if it did not persist beyond the 24 hour period this decrease in glycogen content of the muscle tissue could be attributed to the presence of this slightly increased epinephrine level. At the same time as the muscle glycogen level is low the effects of the hormones on the liver glycogen level would be negligible or at least masked inasmuch as the evidence seems to indicate that the membrane associated adenyl cyclase, possibly enhanced by glucagon, is the responsible factor in early glycogen loss in the liver following partial hepatectomy.

Further research with the controls of Fisher et al. (1971) is necessary with parabiotic rats to allow any firm conclusions to be formed. Since they were using DNA synthesis as the monitoring mechanism

for related activity in the intact rat, no data is available to make further comparisons for glycogen levels. If in this potential research the glycogen level were found to diminish in the intact rat liver following partial hepatectomy of the partner, its breakdown could be attributed to either the hormones or the PBF which was concluded to have affected the DNA synthesis (Fisher et al., 1971). Further analysis to determine the duration and degree of glycogen loss in the liver of the intact rat would help pin-point the factor causing glycogen reduction.

CONCLUSIONS

Based on the data collected and previous available research, I would attribute the decrease in glycogen level in the Quadriceps Femoris following partial hepatectomy to the slight increase in the epinephrine level during this period of time. If further research substantiates the conclusions of Fisher et al. (1971) that the portal blood factor (PBF) is a normal constituent of the blood which causes restorative responses in the liver only when the parenchymal cell-PBF ratio is disrupted, the PBF should have a continuous affect on muscle glycogen at all times regardless of the status of the liver. One exception might be if the parenchymal-PBF ratio disruption were an activating mechanism of an inactive form of the PBF. This does not appear to be the case since the restorative affects including lowered glycogen levels are exhibited in the liver for a much longer period of time than they are in the muscle tissue. If the determining factor of regeneration were the direct cause of the glycogen decrease in the muscle tissue one would expect the muscle glycogen level to mirror the regenerating liver glycogen level more than it does.

The glycogen level at day 4, exceeding normal (Figure 1), remains as an unresolved problem. The possibility exists that it may be considered a type of"overshoot" resulting from the rapid recovery rate, due to individual variation of the rat, or rather that an error in sampling occured. Although no explanation is available for this sharp rise in glycogen level it must not be discounted and may be the focus of further study.

As has been indicated, much additional research is necessary to begin to fully comprehend the determining factor of liver regeneration following partial hepatectomy and the role it plays in the diminished glycogen level in the muscle tissue for a brief period of time.

LITERATURE CITED

Barbiroli, B., and V. R. Potter. 1971. DNA Synthesis and interaction between controlled feeding schedules and partial hepatectomy in rats. Science, 172:738-41

Becker, F. F., and M. W. Bitensky. 1969. Glucagon and epinephrine responsive adenyl cyclase activity of regenerating rat liver. Proc. Soc. Exp. Biol. Med., 130(3):983-86.

Brues, A. M., D. R. Drury, and M. C. Brues. 1936. A quantitative study of cell growth in regenerating liver. A. M. A. Arch. Pathol., 22:658-673.

Bucher, N. L. R. 1963. Regeneration of mammalian liver. Intern. Rev. Cytol., 15:245-300.

Bucher, N. L. R., J. F. Scott, J. C. Aub. 1951. Regeneration of the liver in parabiotic rats. Cancer Res., 11:457-65.

Colowick, S. P., and N. O. Kaplan. 1957. Methods in Enzymology, Vol III. Academic Press Inc., New York. 571 p.

Christensen, B. G., and E. Jacobsen. 1949. Studies on liver regeneration. Acta Med. Scand., (Suppl. 234):103-08.

Davis, J. M. G. 1962. Ultrastructure of rat liver cell cytoplasm during the process of regeneration after partial hepatectomy. Acta Radiol., 58:17-32.

Eckman, C., and H. Holmgren. 1949. The effect of alimentary factors on liver glycogen rhythm and the distribution of glycogen in the lobule. Anat. Rec., 104(2):189-216.

Fisher, B., P. Szuch, M. Levine, and E. R. Fisher. 1971. A portal blood factor as the humoral agent in liver regeneration.

Science, 171:575-77.

Glinos, A. D. 1958. The mechanism of liver growth and regeneration. p. 813-42. In W. D. McElroy and B. Glass, The Chemical Basis of Development. Johns Hopkins Press, Baltimore, Maryland.

Harkness, R. D. 1952. Changes in the liver of the rat after partial hepatectomy. J. Physiol., London. 117:267-77.

Harkness, R. D. 1957. Regeneration of the liver. Brit. Med. Bull., 13:87-93.

Higgins, G. M., and R. M. Anderson. 1931. Experimental pathology of the liver: restoration of the liver of the white rat following partial surgical removal. A. M. A. Arch. Pathol., 12:186-202.

Islami, A. H., G. T. Pack, and J. C. Hubbard. 1959. The humoral factor in regeneration of the liver in parabiotic rats. Surg. Gynecol. Obstet., 108:549-54.

Jaffe, J. J. 1954. Diurnal mitotic periodicity in regenerating rat liver. Anat. Record, 120:935-54.

Leduc, E. 1964. Regeneration of the liver, p. 63-89. In C. Rouiller, The Liver. Vol. 2, Academic Press, New York.

Milne, L. S. 1909. The histology of liver tissue regeneration. J. Pathol. Bacteriol., 13:127-60.

Rogers, A. E., J. A. Shaka, G. Pechet, and R. A. MacDonald. 1961. Regeneration of the liver. Absence of a "humoral factor" affecting hepatic regeneration in parabiotic rats. Am. J. Pathol., 39:561-78.

Ruch, T. C., and H. D. Patton. 1965. Physiology and Biophysics. W. B. Saunders Co., Philadelphia. 1242 p.

Steiner, J. W., M. J. Phillips, and K. Miyai. 1964. Ultrastructural and subcellular pathology of the liver. Intern. Rev. Exptl. Pathol., 3:65-167.

Sulkin, N. N. 1943. A study of the nucleus in the normal and hyperplastic liver of the rat. Am. J. Anat., 73:107-25.